Mutational analysis of *TP53*, *PTEN*, *PIK3CA* and *CTNNB1/β-catenin* genes in human herpesvirus 8-associated primary effusion lymphoma

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ABSTRACT

Human herpesvirus 8 (HHV-8)-associated primary effusion lymphoma is a rare non-Hodgkin's lymphoma often associated with Epstein-Barr virus (EBV) infection. Mutations in *TP53*, *PTEN*, *PIK3CA*, *CTNNB1/β-catenin* genes and deletion of CDKN2A-ARF ($p14^{ARF}$-$p16^{INK4a}$) locus were investigated in sixteen primary primary effusion lymphoma tumors and seven primary effusion lymphoma cell lines using PCR and sequencing. *TP53* mutations were detected in one primary primary effusion lymphoma tumor (6.2%) and two primary effusion lymphoma cell lines (28.6%). BC-3 and BCP-1 cell lines showed *PTEN* gene mutations, associated with a loss of PTEN protein expression in both cases. No mutations were detected in *PIK3CA* and *CTNNB1/β-catenin* hotspot sequences. Only BC-3 contained a homozygous deletion of CDKN2A-ARF locus. Although detected at a higher frequency in primary effusion lymphoma cell lines than in primary primary effusion lymphoma tumors, *TP53* and/or *PTEN* mutations, as well as deletion of CDKN2A-ARF locus are uncommon in primary effusion lymphoma, and are found to correlate with the EBV-negative status of primary effusion lymphoma tumors.

Key words: human herpesvirus 8, non-Hodgkin's lymphoma, primary effusion lymphoma, tumor suppressor gene, mutations.

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Introduction

Somatic mutations of tumor suppressor genes and oncogenes are among the most common genetic alterations found in human malignancies. Moreover, single nucleotide polymorphisms (SNP) in genes involved in apoptosis or cell cycle regulation have been shown to correlate with an increased risk of cancer development, an accelerated cancer onset, a poor response to treatment or a shorter survival. In this setting, a common SNP (rs1042522) in exon 4 of the *TP53* gene, resulting in either Arginine or Proline at codon 72 of the proline-rich domain, has been reported to influence the ability of TP53 protein to induce apoptosis, the Arg72 variant being the most efficient apoptosis inducer.[1] The SNP309 t/g (rs2279744) polymorphism identified in the promoter of MDM2 oncogene, which encodes the negative regulator of TP53, has been shown to influence DNA binding affinity of the transcriptional activator Sp1, leading to changes in MDM2 expression levels and attenuation of TP53 response. In sporadic cancers, including diffuse large B-cell lymphomas (DLBCL), the SNP309 g/g genotype has been found to correlate with an earlier age of tumor onset in female patients.[2] In other studies however, neither MDM2 SNP309 nor TP53 SNP72 have been found to be associated with survival or age of tumor onset in patients with non-Hodgkin's lymphoma (NHL).[3,4]

Primary effusion lymphoma (PEL) is a rare NHL which usually develops as lymphomatous effusions in the serous cavities of immunocompromised patients, especially Human Immunodeficiency Virus type-1 (HIV-1)-infected individuals and solid organ transplant recipients.[5,6] PEL tumor cells display pleiomorphic morphology and frequently lack B-cell lineage antigen expression, despite their B-cell monoclonal origin. These cells are latently infected with HHV-8, and are in most cases co-infected with Epstein-Barr virus (EBV).[7,8] The phos-

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phatidylinositol 3'-kinase (PI3K)/AKT cascade has been identified as constitutively activated in PEL and critical for cell survival.[9] This pathway is negatively regulated by the non-redondant lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10). Among the best known genetic alterations leading to the constitutive activation of PI3K/AKT cascade, the loss of *PTEN* tumor suppressor gene and the activating

Table 1. Analysis of mutations in oncogenes and tumor suppressor genes.

			TP53		*PTEN*		*PIK3CACTNNB1*/CDKN2A-ARF			
								β-catenin (p14^{ARF}-p16^{INK4a})		
Sample	Diagnosis	EBV	Status	Nucleotide/amino acid substitution	Status	Nucleotide/amino acid substitution	Status	Status	Status	Nucleotide/amino acid substitution
Primary PEL cases n.										
551	AIDS-PEL	+	wt		wt		wt	wt	wt	
718	AIDS-PEL	+	wt		wt		wt	wt	wt	
809	AIDS-PEL	+	wt		wt		wt	wt	wt	
820	HIV-1-negative PEL	–	wt		wt		wt	wt	wt	
895	AIDS-PEL	+	wt		wt		wt	wt	wt	
942	AIDS-PEL	+	wt		wt		wt	wt	wt	
974	HIV-1-negative PEL	–	wt		wt		wt	wt	wt	
977	AIDS-PEL	–	wt		wt		wt	wt	wt	
990	AIDS-PEL	–	wt		wt		wt	wt	wt	
993	AIDS-PEL	+	wt		wt		wt	wt	wt	
1006	AIDS-PEL	–	M-Ex9	c991g / Q331E	wt		wt	wt	wt	
1014	AIDS-PEL	+	wt		wt		wt	wt	wt	
1018	AIDS-PEL	+	wt		wt		wt	wt	wt	
1205	AIDS-PEL	+	wt		wt		wt	wt	wt	
1298	Pt-PEL	–	wt		wt		wt	wt	wt	
1377	Pt-PEL	–	wt		wt		wt	wt	wt	
PEL cell lines										
BC-2		+	wt		wt		wt	wt[2]	wt	
BC-3		–	wt		M-Ex7	Heterozygous del 740-741 ta/Nonsense 250	wt	wt[2]	Ex1 to 2	Homozygous deletion / absence of protein[3]
BCBL-1		–	M-Ex7 M-Ex8	g738a / M246I [1] g>a splice acceptor	wt		wt	wt	wt	
BCP-1		–	M-Ex7 M-Ex7	a736g / M246V g775a / D259N	Ex6 to 9	Homozygous deletion / absence of protein	wt	wt	wt	
BBG-1		+	wt		wt		wt	wt	wt	
ISI-1		-	wt		wt		wt	wt	wt	
CRA-BCBL		+	wt		wt		wt	wt	wt	
ALL cell lines										
CEM		-	M-Ex5 M-Ex7	g524a / R175H g743a / R248Q	Ex2 to 5	Homozygous deletion/ absence of protein	wt	wt	Ex1 to 2	Homozygous deletion / absence of protein
DAUDI		+	M-Ex6 M-Ex8	c637t / R213* g797a / G266E	M-Ex6	t524g / V175G	wt	wt	wt	
RAJI		+	M-Ex6 M-Ex7	g638a / R213Q t700c / Y234H	wt		wt	wt	wt	
REH		–	wt		wt		wt	wt	Ex1 to 2	Homozygous deletion / absence of protein
RS(4;11)		–	M-Ex6	Ins 467c/ Nonsense 180	wt		wt	wt	Ex1 to 2	Homozygous deletion / absence of protein
Healthy controls' PBMCs										
H7			wt		wt		wt	wt	wt	
H9			wt		wt		wt	wt	wt	
H22			wt		wt		wt	wt	wt	
H27			wt		wt		wt	wt	wt	

AIDS: acquired immune deficiency syndrome; ALL: acute lymphoblastic leukemia; Del: deletion; EBV: Epstein-Barr virus; Ex: exon; Ins: insertion; M: mutation; PBMCs: peripheral blood mononuclear cells; PEL: primary effusion lymphoma; PIK3CA: phosphatidylinositol 3'-kinase p110 catalytic subunit; Pt: post-transplantation; PTEN: phosphatase and tensin homolog deleted on chromosome 10; wt: wild type. [1]Previously reported;[17] [2]Previously reported;[14] [3]Previously reported.[16]

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mutations of the p110 catalytic subunit of PI3K (PIK3CA) have been reported in many cancers including NHL.[10-13] PEL tumor cells have been shown to express high levels of β-catenin, which is a downstream activator of the Wnt signaling pathway.[14] Mutations in exon 3 of the *CTNNB1/β-catenin* gene resulting in the accumulation of β-catenin in the cytoplasm, have been found in several cancer types, including lymphoproliferative disorders developed in renal transplant recipients.[15] A loss of CDKN2A/p16^{INK4a} protein expression has been reported in all primary PEL isolates analyzed. However, the molecular events leading to CDKN2A/p16^{INK4a} gene silencing have only been identified in a few PEL cell lines.[16] Since mutations of *PTEN, PIK3CA, CTNNB1/β-catenin* genes and deletion of CDKN2A-ARF (p14^{ARF}-p16^{INK4a}) locus had not been previously investigated in primary PEL tumors, we performed an extensive molecular analysis of mutations and SNP in a large series of PEL samples.

Design and Methods

The study included seven PEL cell lines and sixteen primary tumor samples (seven pleural effusions, eight ascites, one pericardial effusion) collected from 12 HIV-1-infected patients, 2 HIV-1-negative elderly individuals and 2 renal transplant recipients with HHV-8-associated

Table 2. Analysis of single nucleotide polymorphisms in several genes involved in cell cycle regulation.

Sample	Age (yr)* / Sex / Ethnicity	HIV	CD4 count (x10^9/l)* / HIV RNA* (copies/ml)	MCD	EBV status of PEL	Outcome / Survival from PEL diagnosis	TP53 SNP72	TP53 Ins16bp	MDM2 SNP309	CDKN1A S31R	CDKN1A 3'UTR (c70t)	CDKN1B V109G	CDKN1B 5'UTR (c79t)	CCND1 g870a	CCND3 A259S	STK15 t91a (F31I)	CDC25C R70C	CDC2L1 A655V	CDC6 I441V
Primary PEL cases n.																			
551	38 / M / C	yes	136 / ND	no	+	D / 4 wk	Pro/Pro	- / -	t/t	Ser/Ser	c/c	Val/Val	ct	a/a	Ala/Ser	a/t	Arg/Arg	Ala/Ala	Val/Val
718	36 / M / C	yes	264 / 214,000	no	+	D / 7 wk	Arg/Pro	- / -	t/t	Ser/Ser	c/c	Val/Val	cc	a/g	Ala/Ser	a/t	Arg/Arg	Ala/Ala	Ile/Val
809	43 / M / C	yes	17 / 130,000	no	+	D / 16 wk	Arg/Arg	- / -	t/t	Ser/Ser	c/c	Val/Val	tt	g/g	Ala/Ser	a/a	Arg/Cys	Ala/Ala	Val/Val
820	78 / M / C	no	255 / -	no	-	D / 6 wk	Arg/Arg	- / -	t/t	Ser/Ser	c/c	Val/Val	tt	g/g	Ala/Ala	a/a	Arg/Cys	Ala/Ala	Val/Val
895	43 / M / C	yes	74 / 175,000	no	+	D / 11 wk	Arg/Arg		t/t						Ser/Ser				Val/Val
942	37 / M / C	yes	130 / <200	no	+	D / 29 mo	Arg/Pro		t/t	Ser/Ser			ct	a/g		a/t			
974	86 / F / C	no	ND / -	yes	-	D / 8 wk	Arg/Arg	- / -	t/g	Ser/Arg	c/t	Val/Gly	cc	a/g	Ala/Ala	a/a	Arg/Cys	Ala/Ala	Ile/Val
977	48 / M / C	yes	73 / <200	yes	-	D / 9 mo	Arg/Arg	- / -	t/t	Ser/Ser	c/c	Val/Gly	cc	a/g	Ala/Ser	a/t	Arg/Arg	Ala/Ala	Ile/Ile
990	78 / M / C	yes	391 / <200	no	-	CR / >5 yr	Arg/Pro	- / ins	t/g	Ser/Ser	c/c	Val/Gly	cc	a/a	Ala/Ser	a/a	Arg/Cys	Ala/Ala	Val/Val
993	37 / F / A	yes	204 / 280,000	yes	+	D / 42 mo	Arg/Arg	- / -	t/t	Ser/Arg	c/t	Gly/Gly	tt	a/a	Ala/Ser	a/a	Arg/Cys	Ala/Val	Val/Val
1006	48 / M / C	yes	280 / <200	yes	-	D / 41 mo	Arg/Pro		t/t	Ser/Ser	c/t	Val/Gly	cc	g/g		a/a			Val/Val
1014	38 / M / C	yes	137 / <200	yes	+	D / <1 wk	Arg/Pro	- / -	t/t	Ser/Ser	c/c	Val/Val	ct	a/a	Ala/Ser	a/t	Arg/Cys	Ala/Ala	Val/Val
1018	44 / M / C	yes	210 / 160,000	no	+	D / 2 wk	Arg/Pro	- / ins	t/t	Ser/Ser	c/c	Val/Val	ct	a/g	Ala/Ser	a/t	Arg/Cys	Ala/Val	Ile/Val
1205	45 / M / A	yes	36 / 153,000	yes	+	D / 5 wk	Pro/Pro	- / ins	t/t	Ser/Arg	c/t	Gly/Gly	cc	a/a	Ala/Ser	a/a	Arg/Arg	Ala/Val	Ile/Ile
1298	57 / M / A	no	77 / -	no	-	D / 8 mo	Arg/Pro	- / ins	t/t	Ser/Ser	c/c	Gly/Gly	ct	a/a	Ala/Ala	a/t	Arg/Cys	Ala/Val	Ile/Val
1377	63 / M / A	no	ND / -	no	-	D / 1 wk	Pro/Pro	- / -	t/t	Ser/Arg	c/c	Gly/Gly	tt	a/a	Ala/Ala	a/a	Cys/Cys	Ala/Val	Ile/Val
PEL cell lines																			
BC-2							Arg/Pro	- / -	t/t	Ser/Ser	c/c	Val/Gly	ct	a/a	Ser/Ser	a/a	Arg/Arg	Ala/Ala	Val/Val
BC-3							Arg/Arg	- / -	g/g	Ser/Ser	c/c	Val/Val	cc	a/a	Ser/Ser	a/a	Arg/Arg	Ala/Ala	Val/Val
BCBL-1							Pro/Pro	- / ins	g/g	Ser/Ser	c/c	Val/Gly	cc	a/g	Ala/Ser	a/a	Arg/Arg	Ala/Ala	Val/Val
BCP-1							Arg/Arg	- / -	g/g	Ser/Ser	c/c	Val/Val	cc	a/a	Ser/Ser	a/a	Arg/Cys	Ala/Ala	Val/Val
BBG-1							Arg/Pro	- / ins	t/g	Ser/Ser	c/c	Val/Gly	ct	a/a	Ala/Ser	a/a	Arg/Cys	Ala/Ala	Ile/Val
ISI-1							Arg/Pro	- / ins	t/t	Ser/Arg	c/t	Val/Val	ct	g/g	Ala/Ser	a/a	Cys/Cys	Ala/Ala	Val/Val
CRA-BCBL							Pro/Pro	- / -	t/t	Ser/Ser	c/c	Val/Val	ct	a/a	Ala/Ser	a/t	Arg/Arg	Ala/Ala	Val/Val
ALL cell lines																			
CEM							Arg/Arg	- / -	t/t	Ser/Ser	c/c	Val/Gly	ct	a/g	Ala/Ser	a/t	Arg/Cys	Ala/Ala	Val/Val
DAUDI							Arg/Arg	- / ins	t/t	Ser/Arg	c/t	Gly/Gly	cc	g/g	Ala/Ala	a/a	Arg/Cys	Ala/Val	Val/Val
RAJI							Arg/Pro	- / -	t/t	Ser/Arg	c/t	Gly/Gly	tt	a/a	Ala/Ala	a/t	Arg/Arg	Ala/Val	Ile/Val
REH							Arg/Arg	- / -	t/t	Ser/Ser	c/c	Val/Val	cc	g/g	Ala/Ser	a/a	Cys/Cys	Ala/Ala	Ile/Val
RS(4;11)							Arg/Arg	- / -	t/g	Ser/Ser	c/c	Val/Val	cc	a/g	Ser/Ser	a/a	Arg/Arg	Ala/Ala	Ile/Val

*At the time of PEL diagnosis; A: African; C: Causasian; CR: complete remission; D: death; EBV: Epstein-Barr virus; F: female; HIV: human immunodeficiency virus type-1; M: male; MCD: HHV-8-associated multicentric Castleman disease; mo: months; ND: not determined; PEL: primary effusion lymphoma; SNP: single nucleotid polymorphism; wk: weeks; yr: years;

favourable genotype | intermediate genotype | unfavorable genotype

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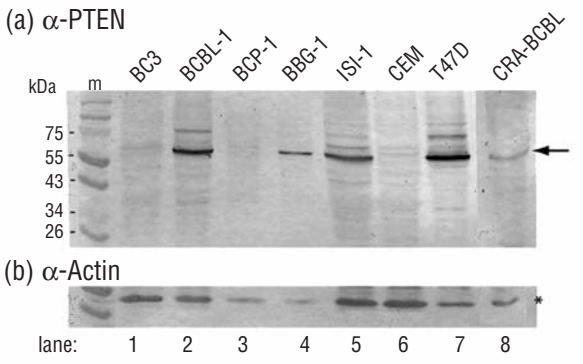

Figure 1. PTEN expression in PEL cell lines. Lysates from BC-3 (lane 1), BCBL-1 (lane 2), BCP-1 (lane 3), BBG-1 (lane 4), ISI-1 (lane 5) and CRA-BCBL (lane 8) cell lines were analyzed by Western blot for PTEN (A) and actin (B) protein expression. The T-ALL CEM (lane 6) and breast adenocarcinoma T47D (lane 7) cell lines served as negative and positive controls, respectively. Sodium dodecyl sulfate (SDS)-denatured cellular proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide gels using a discontinuous buffer system (Laemmli, U.K., 1970). The transfer of proteins onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences) was carried out using a semi-dry blotting system. Blots were blocked with skimmed milk in TNT buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) and developed after successive incubation with anti-human PTEN rabbit antibody (R&D Systems) and alkaline phosphatase-labeled goat anti-rabbit IgG conjugate (Sigma-Aldrich).

PEL. All patients samples (Tables 1 and 2) were collected in accordance with the ethical regulations of our institution, as indicated in our previous studies.[5,6] The clinical data were collected from the patients' records by the same examiner (*EB*). Human acute lymphoblastic leukemia (ALL) cell lines of T (CEM) and B [DAUDI, RAJI, REH, RS(4;11)] cell lineage origins, and peripheral blood mononuclear cells (PBMCs) from Caucasian healthy donors, were used as controls. Mutations of *TP53* (exons 4-11), *PTEN* (exons 1-9), *PIK3CA* (exons 9 and 20), *CTNNB1/β-catenin* (exon 3) genes and deletion of CDKN2A-ARF ($p14^{ARF}$-$p16^{INK4a}$) locus were detected by PCR and direct sequencing. SNP in several genes involved in apoptosis and cell cycle regulation including SNP72 and ins16bp in TP53, SNP309 in MDM2, S31R and 3'UTR (c70t) in CDKN1A/$p21^{Cip1}$, V109G and 5'UTR (c79t) in CDKN1B/$p27^{Kip1}$, g870a in CCND1/cyclin D1, A259S in CCND3/cyclin D3, F31I (t91a) in STK15/aurora A, R70C in CDC25C, A655V in CDC2L1 and I441V in *CDC6* genes, were assessed by PCR.

Results and Discussion

As previously observed,[7,8] the frequency of *TP53* gene mutations in PEL was found to be low, as they were detected in only one out of sixteen (6.2%) tumor samples and in two out of seven cell lines (28.6%, Table 1). In accordance with previous reports,[17] BCBL-1 was found to harbor a heterozygous M246I mutation of *TP53*. BCP-1 contained two missense mutations leading to single nucleotide changes (M246V and D259N) in both alleles of exon 7. Only BC-3 contained a homozygous deletion of CDKN2A-ARF locus, in agreement with previous reports.[16] No mutations were found in PIK3CA and CTNNB1/β-catenin hotspot sequences. PTEN gene alterations were identified in two PEL cell lines (Table 1). BC-3 carried a monoallelic 2 bp-deletion in exon 7 leading to a frameshift at codon 247 followed by a stop at codon 250, whereas BCP-1 harbored a homozygous deletion of PTEN exons 6 through 9. In both cases, these mutations resulted in the loss of PTEN protein expression (Figure 1). Approximately 20% of high-grade B-cell NHL have TP53 mutations[18] whereas the reported rates of *PTEN* and *PIK3CA* mutations are around 5%[10,11] and from 1 to 8%,[12,13] respectively. By contrast, *CTNNB1/β-catenin* gene mutations occur more frequently in T-cell or NK/T-cell NHL from Asian patients than in B-cell NHL.[15] Our results indicated that these genetic alterations occur at a lower frequency in PEL than in other subtypes of B-cell NHL, and suggested that other mechanisms may be relevant in activating oncogenic pathways.

Among HHV-8 lytic proteins with transforming potential, G-protein coupled receptor (vGPCR)[19] and K1[20] have been shown to constitutively activate the PI3K/AKT pathway, and viral interferon regulatory factor 1 (vIRF1) to inhibit TP53 function.[21] LANA-1, which is constitutively expressed in tumor PEL cells, is able to suppress TP53 function and to induce β-catenin accumulation by trapping the glycogen synthase kinase-3b (GSK-3b) into the nucleus.[14] Beside genetic alterations of coding sequences, additional mechanisms might participate in the loss of tumor suppressor gene function such as epigenetic silencing. In BCBL-1, BCP-1 and ISI-1 cell lines, gene hypermethylation has been identified as underlying the loss of CDKN2A/$p16^{INK4a}$ locus expression.[16] Post-translational modifications like phosphorylation can lead to PTEN inactivation, as observed in Hodgkin's lymphoma cell lines.[22] Because K1 expression has been shown to increase PTEN phosphorylation in transfected BJAB cells, it might contribute to the constitutive activation of PI3K/AKT pathway observed in PEL.[20] Like in DLBCL and Burkitt's lymphomas, TP53 and PTEN gene alterations occurred at a higher frequency in PEL cell lines (3/7) than in primary tumors (1/16). Considering both PEL cell lines and primary PEL tumors, the mutation rate was found to be significantly higher in EBV-negative PEL (4/11) compared to EBV-positive PEL (0/12, p=0.037, Fisher's test). However, this difference was not significant when only the primary PEL cases were analyzed. A similar correlation between the presence of TP53 gene mutations and the absence of EBV has been reported in Hodgkin's lymphomas,[23] although this result could not be confirmed by further studies.[24]

Considering the 16 patients with PEL (Table 2), the CDKN1A/$p21^{Cip1}$ S31R, CDKN1B/$p27^{Kip1}$ V109G and CDC2L1 A655V polymorphims were found to be significantly associated with an African origin (p=0.033, 0.002 and 0.007, respectively), the CDKN1A/$p21^{Cip1}$ c70t polymorphism with the presence of an HHV-8-associated multicentric Castleman disease (p=0.015) and the

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CCND3 A259S polymorphism with HIV-1 infection and EBV status of PEL (p=0.001 and 0.015, respectively). However, no correlation could be found between these SNP, the age of patients at the time of PEL diagnosis and their survival from the date of PEL diagnosis.

Our results confirm that mutations of *TP53* and *PTEN* tumor suppressor genes, as well as deletion of CDKN2A-ARF locus, are uncommon in PEL, although they were detected at a higher frequency in PEL cell lines than in primary PEL tumors. Moreover, these genetic alterations were found to be restricted to EBV-negative PEL tumors. No mutations were detected in PIK3CA and CTNNB1/β-catenin hotspot sequences, suggesting that other mechanisms are involved in the pathogenesis of HHV-8-associated PEL.

Authorship and Disclosures

EB was the principal investigator and takes primary responsibility for the paper. EB recruited the patients. EB, AM, SSH and PP performed the laboratory work for the study. EB and PP performed the statistical analyses and wrote the paper.

The authors reported no potential conflicts of interest.

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